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Flameless Atomic Absorption (FAA) and Gas–Liquid Chromatographic Studies in Arsenic Bioanalysis

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Procedures for assessment of arsenic in soft tissue by use of flameless atomic absorption (FAA) and gas–liquid chromatography (GLC) have been evolved, with special emphasis on the analytical distinction among inorganic, monomethyl-, and dimethylarsenic in several oxidation states.

The chemical bases for such speciation reside in several properties of the arsenicals under consideration: (1) pentavalent inorganic arsenic, methylarsonic, and cacodylic acid are not extracted from tissue matter made strongly acid with hydrochloric acid, while the corresponding trivalent forms (as halides) are extracted; (2) chloroform extracts of samples treated under reducing conditions (HCl–KI) retain organoarsenicals when these extracts are re-extracted with water, but do not when aqueous solutions of oxidants are employed; (3) reduced cacodylate (dimethylarsinous acid) is not detected in the graphite furnace of an FAA unit under conditions selected, while cacodylate can be so detected.

For GLC studies, monomethyl- and dimethylarsenic are simultaneously measured as the diethyldithiocarbamate complexes with an instrument equipped for electron-capture detection and containing a glass column packed with silanized 5% OV-17 on Anakrom A.S.

Recently, we reported the development of both flameless atomic absorption (FAA) and gas–liquid chromatographic (GLC) techniques for measurement of inorganic and organic arsenic in water and urine (*1, 2*). These approaches employ extraction and chelation-extraction via the iodide derivatives and thus eschew the generation and transfer of the arsenicals as the gaseous hydrides as well as the analytical hazards associated therewith (*3*).

Presently, similar approaches are being directed to assessment of chemically variant arsenicals in mammalian soft tissue and these studies to date comprise the text of this report. To the extent that levels of arsenic in biological media deriving from mammalian origin are such that detection limits need not be quite as critical as those for natural water or ambient air levels, early emphasis has been placed on methods for chemical speciation of sample arsenicals, to be followed by technique refinement where necessary to achieve the requisite sensitivity.

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As part of the FAA studies dealing with arsenicals in tissue, we have developed a method for total arsenic in tissue.

Experimental

FAA Studies of Arsenicals in Tissue

Homogenates of liver, kidney, etc. (10% w/v) were prepared by use of deionized water and acid-washed homogenizing apparatus. Sample volumes (0.5 ml) were freeze-dried along with appropriate matrix standards (0.2–1.0 ppm added to homogenate from a pool of control animal tissue). Freeze-dried homogenates were them employed for assessment of total as well form-variable arsenic.

Total Arsenic in Soft Tissue. Lyophilized samples were wet-ashed in a two-stage sequence by use of ultra-pure acids, acid-washed 1-dram vials (Kimbleware), and acid-washed boiling beads. A 0.4-ml portion of concentrated ultra-pure nitric acid and 0.05 ml ultra-pure sulfuric acid were added and the mixture heated to near dryness. The samples were cooled, 0.2 ml of the nitric acid was added, and this step was repeated. To the cooled residues were added low-arsenic hydrochloric acid (8*N*, 0.3

ml) and 0.1 ml saturated potassium iodide solution followed by 2.0 ml of chloroform and 0.1 ml of freshly prepared aqueous 2% diethylammonium diethyldithiocarbamate solution.

Extraction of arsenic diethyldithiocarbamate into chloroform via maximum agitation on a Vortex-Genie apparatus for 15 sec was followed by re-extraction of a portion of the organic layer (1.0 ml) into a volume (1.0 ml) of dilute (2*N*) nitric acid solution. Aliquots of the aqueous phase (10–30 μl) were then inserted into the graphite tube of the furnace accessory of an atomic absorption spectrometer. Specific conditions, where the Perkin-Elmer Model 2100 furnace accessory is employed, were: dry, 20 sec at 100°C, char, 20 sec at 500°C; atomize, 15 sec at 2100°C.

Chemically Variant Arsenicals in Soft Tissue. Trivalent arsenic may be prepared by the hydrolysis of pure samples of arsenic (III) chloride added to deionized water in volumetric flasks or the dissolution of pure arsenic trioxide in hydrochloric acid followed by deionized water dilution. Pentavalent arsenic, as the free acid, may be used as obtained from commercially available arsenic AA standards. Cacodylic acid, as the free acid, and methylarsonic acid, as the disodium salt, are commercially available. Methylarsonous and dimethylarsinous acids may be prepared via dilute alkali hydrolysis of the iodides followed by solution acidification.

Samples of freeze-dried tissue homogenate in 1-dram acid-washed vials were treated under both nonreducing (hydrochloric acid) and reducing conditions as noted below. For the former, 0.5 ml of concentrated hydrochloric acid was added, followed by 0.2 ml of deionized water, while reducing media were generated by using this volume of acid but with the addition of potassium iodide solution (saturated, 0.2 ml). Samples were set aside for 1 hr at room temperature with occasional shaking. Extraction of arsenicals from both types of media was achieved via 2.0 ml of benzene equilibrated over hydrochloric acid, the mixtures being agitated at moderate speed by using a Vortex-Genie apparatus for 45–60 sec. Centrifugation was carried out at 2900 rpm for 15 min. Emulsion formation, if a problem, could be minimized by adding an additional 0.3 ml of deionized water to the sample without serious effect on extraction efficiency.

Aliquots of the benzene layer (1.0 ml) were extracted against 0.5 ml of either deionized water or 2*N* nitric acid, the former fraction giving signals due to inorganic and monomethylarsenic while inorganic arsenic, mono- and dimethylarsenic contribute to the signal in the nitric acid fraction. The deionized water extract was acidified with hydrochloric acid and potassium iodide added, the mixture of iodides being then extracted into 1.0 ml of chloroform. Re-extraction of the chloroform layer with 0.5 ml deionized water, furnished inorganic arsenic in the aqueous phase.

For purposes of quantitation, inorganic arsenic was measured directly and quantitated via the matrix standard samples. Subtraction of the signal in the final deionized water layer from that initially obtained (benzene) gave the amount of monomethylarsenic by relating to the standards. Similarly, subtraction of the first deionized water extract signal from that with dilute nitric acid gave the amount of dimethylarsenic (cacodylic acid) present.

Gas–Liquid Chromatographic (GLC) Studies

Samples of freeze-dried homogenates were worked up initially in identical fashion to those for speciation via FAA spectrometry, except that chromatographic-grade benzene was employed. Volumes of the benzene layer (1.0 ml) were then transferred to a second set of 1-dram vials containing 0.5 ml of 4–6*N* sulfuric acid, 0.1 ml of potassium iodide solution and 0.05 ml of dilute sodium metabisulfite solution. After addition of freshly prepared diethylammonium diethyldithiocarbamate solution, the mixture was agitated for 30 sec with a Vortex-Genie apparatus and centrifuged. The benzene layers were carefully transferred to vials containing 0.5 ml of 0.5*N* sodium hydroxide solution and reshaken. A portion of the benzene layer was transferred to vessels containing anhydrous sodium sulfate and aliquots (2–10 μl) injected into a gas chromatograph equipped for electron-capture detection (^{3}H foil) and having a 4-ft glass column, packed with heavily silanized 5% OV-17 on Anakrom A. S. Quantitation of the peaks for mono- and dimethylarsenic diethyldithiocarbamate was carried out in the usual manner by using the matrix standards.

Results and Discussion

FAA Studies

In a recent report, Reinke et al. (*4*) described the successful use of a procedure for assessment of both arsenic(III) and arsenic(V) in samples of fish tissue via acid treatment, extraction with benzene and the use of cooper(I) ion for reduction of pentavalent arsenic to the benzene-extractable trivalent form. We have systematically studied their tech-

nique with reference to soft tissue and FAA analysis and have elaborated upon this approach to include mono- and dimethylarsenic.

In this regard, the extraction behavior of the oxidized/reduced pairs: penta- and trivalent arsenic, methylarsonic and methylarsonous acids, cacodylic and dimethylarsinous acids, was studied.

Hydrochloric acid at varying concentration was chosen as sample medium, acid treatment being carried out at room temperature for 1 hr. We desired an acid medium having sufficient hydrolytic activity to release the arsenicals of interest from their bound biomolecular forms for ready extraction but having little or no effect on the carbon–arsenic bonds in the methylated arsenicals.

Soft tissue samples were handled as homogenates (10%) made up in deionized water and then freeze-dried to avoid the dilution of acid reagent in the bound-arsenic liberation step. Recovery studies with the use of various arsenicals indicated that freeze drying was without effect on those arsenicals of analytical interest in this report.

The acid medium could be rendered chemically reducing by the addition of a volume of potassium iodide, which converts pentavalent arsenicals to their corresponding trivalent forms. Where no reducing activity was desired, a volume of deionized water was employed.

Acid treatment in this fashion for 1 hr at room temperature was followed by benzene extraction ($\geq$ 2:1) and re-extraction into aqueous phases. Pentavalent inorganic arsenic, methylarsonic, and cacodylic acids do not undergo extraction under conditions of straight hydrochloric acid treatment, while the use of iodide as reducing agent or the direct addition of trivalent arsenicals under nonreducing conditions yielded essentially quantitative extraction and recovery data. In Figure 1 are depicted representative spectrograms for extracts (aqueous re-extraction of benzene layers) obtained from freeze-dried homogenates to which monomethyl- and dimethylarsenic, in pentavalent and trivalent forms, have been added.

At this stage of our studies, we made the interesting observation that dimethylarsinous acid, a reduced form of cacodylic acid, is not seen in the furnace accessory in the atomic absorption instrument under conditions being employed, particularly the charring temperature used. Cacodylic acid itself gives a prominent signal at equivalent concentration. By conversion of the water extracts to an oxidizing medium (2.0*N* nitric acid) or carrying out the re-extraction directly with 2*N* oxidant, generation of an instrument-detectable species is achieved.

At this point, the following analytical factors

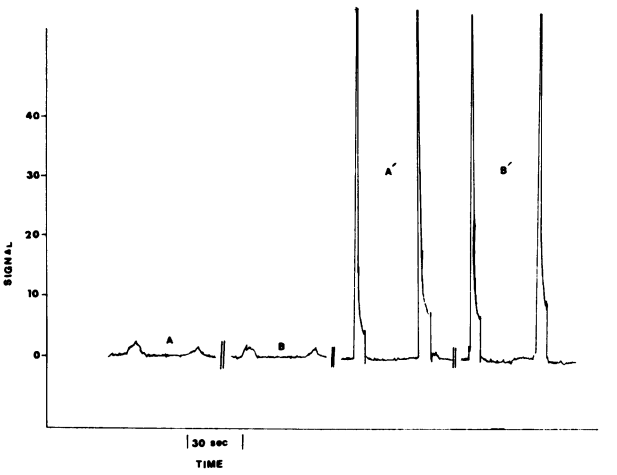

FIGURE 1. FAA spectrometric analysis of aqueous extracts of methylated arsenicals in different oxidation states: (A) methylarsonic acid; (B) dimethylarsinic (cacodylic) acid; (A′) methylarsonous acid, added as such or generated by reduction; (B′) dimethylarsinous acid, isolated in oxidizing medium from benzene extract.

pointing to a separation scheme of value in arsenic chemical speciation were established. (1) From our earlier studies involving arsenical analysis in urine and water (*1, 2*), chloroform extracts of acidified, iodide-treated samples which contain mixtures of arsenicals as the iodides (inorganic, monomethyl- and dimethylarsenic), surrender only inorganic arsenic to a deionized water phase but yield all arsenicals when the process is carried out with an oxidant solution (dilute dichromate). (2) Pentavalent arsenicals are not extracted from hydrochloric acid media, while the trivalent forms are, whether the latter are originally present as such or generated via reduction. (3) Dimethylarsinous acid is not detected in the instrument under conditions employed, while treatment with an oxidant does permit arsenic signal generation.

A separation flow scheme based on the above is depicted in Figure 2. That portion of the figure above the dotted line involves a nonreducing medium and hence, isolation steps here would involve trivalent arsenicals originally present as such in the sample. The corresponding portion below the line, entailing reduction of pentavalent forms to their trivalent states, furnishes fractions possessing total levels of trivalent arsenicals.

Fractions A and A′ contain all three arsenicals being studied, but arsenic signals arise as the sum of only inorganic and monomethyl arsenic, demethylarsinous acid not being detected. Fractions B and B′, via direct extraction with 2*N* nitric acid or acidification of A and A′, generate a total arsenic signal. Fractions C and C′, involving the intermedi-

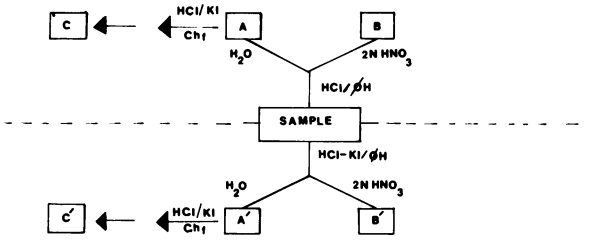

FIGURE 2. Analytical separation scheme for inorganic and methlyated arsenicals in pentavalent and trivalent oxidation states by using FAA spectrometry.

acy of chloroform reextraction, contain only inorganic arsenic.

In both portions of the flow scheme, reducing and nonreducing, two of the three arsenical levels are being assessed by difference (in signal amplitude) while differences in the signal amplitude A' - A, B' - B, and C' - C permit distinction as to at least two oxidation states.

Since much of data being gathered entails differences in signal amplitude, good precision (low variance) is mandatory, and we have assessed the various factors that bear heavy influence on this. The volume of analyte delivered to the graphite furnace is of significance in precision, in our hands the use of volumes greater than 20 μl (30–40 μl) improving precision considerably. The chemical form of the standards is important, salts of the arsenicals yielding somewhat higher signal amplitudes than the corresponding amount delivered as the free acid. The routine use in our laboratory of matrix standards, control tissue with little or no arsenic and containing added arsenic in the various chemical forms minimizes this problem.

Some preliminary data, shown in Table 1, indicate that quantitative or near-quantitative recovery for those arsenicals studied are readily achieved, but the precision bears improvement. The tabulated material was gathered using 10 μl aliquots, and we have since observed that increase in volume to 30 μl considerably reduces the relative standard deviation (RSD). Further study of the quantitative aspects of this flow scheme is under way.

While most of our efforts have been directed to studies in arsenic speciation, we have also evolved a method for total arsenic in tissue. We are aware that considerable utility still exists in the analytical community for improved methods of total arsenic measurement in various media. Furthermore, total arsenic levels in samples also being studied for the existence of that element in various chemical forms provide a valuable reference point in terms of (1) arsenicals present but not detected by a particular speciation technique and (2) recovery accuracy of

Table 1. Recovery and precision data for arsenicals in tissue by FAA spectrometry.

Arsenical	Amount added ppm	*N*	Recovery, % (RSD)
Inorganic As	0.8	5	98.6(± 9.5)
Mixture of:			
Inorganic As	0.8	5	97.8(± 8.3)
Methyl-As	0.6	5	91.3(±12.0)

the measured arsenicals from the sample.

A two-step wet ashing sequence of freeze-dried tissue homogenate followed by arsenic chelation-extraction and re-extraction steps are the salient features of the method. The ashing procedure has been carefully evaluated for inorganic, monomethyl-, and dimethylarsenic and the analytical steps as described are those that permit comparable quantitative data for all forms.

A mixture of ultra-pure nitric and sulfuric acids, 0.4 and 0.05 ml respectively, was added to the samples in 1-dram acid-washed vials, and the samples were held at room temperature for 10–15 min followed by heating at the minimum temperature that sustains a moderate boiling rate. After heating to near dryness, ashing was repeated by use of 0.2 ml of nitric acid.

Arsenic was then converted to the trivalent iodide (see Experimental) and extraction into chloroform using diethyldithiocarbamate solution carried out. Re-extraction of the arsenic into an aqueous phase was achieved with dilute (2*N*) nitric acid solution. An oxidizing medium was found necessary to destroy the chloroform-soluble carbamate complex.

In Figure 3 are presented spectrograms for a typical tissue arsenic assay, while Table 2 summarizes the corresponding quantitative data for the method. An optimal recovery of ca. 80% is seen with all three arsenicals studied, with rather good precision of analysis over several ranges of levels.

Table 2. Recovery and precision data for tissue total arsenic by FAA spectrometry.

As added, ppm	*N*	Recovery, % (RSD)
0.5	6	78.5(± 7.2)
0.2	5	79.8(±3.3)

Gas–Liquid Chromatographic Studies

Complementing our interests in the application of FAA spectrometry to chemical speciation of arsenic in biological media are parallel studies employing GLC, a technique widely known for its specificity in analysis.

The feasibility of using GLC in analysis of form-variable arsenicals in liquid media was demonstrated by us in an earlier report (*2*) and entailed the

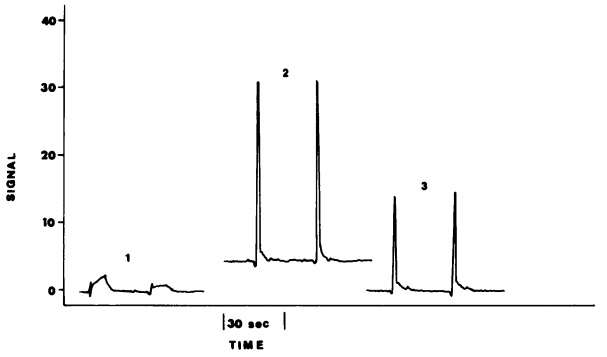

FIGURE 3. Spectrograms for tissue total arsenic by FAA spectrometry; (1) liver homogenate from control animal; (2) control homogenate with added arsenic (0.2 ppm); (3) liver homogenate from rat dosed with inorganic arsenic.

isolation of the arsenicals as the stable diethyldithiocarbamate complexes, followed by chromatographic manipulation on a column containing a heavily silanized silicone packing.

Similar studies involving soft tissue are presently in progress and our results to date are described below.

Preliminary handling of the tissue samples, again as freeze-dried homogenates, was essentially identical to that for the FAA spectrometry techniques noted above, with the notable difference that use of chromatographic-grade benzene was required to minimize interference from chromatographic contaminants present in lower grades of this particular solvent.

For further sample work-up, volumes of the benzene extracts were transferred to vials containing hydroiodic acid and solutions of chelating agent and sodium metabisulfite then introduced. Metabisulfite, a reducing agent, serves to prevent the presence of iodine, the latter reacting with and destroying the complexing efficiency of the chelant. A further transfer of the organic layer, now containing the arsenicals as the diethyldithiocarbamate complexes, to vials containing dilute alkali was done; this step helped to remove chromatographic interferents from tissue originally and, to minimize an effect on EC detector sensitivity due to the generation of sulfur-containing fragments when the chelant is introduced in acid media. This latter effect, interestingly, appears to be a function of EC detector design, being observed in one instrument but not in a second unit.

Subsequent to alkali clean-up, the layers were dried over anhydrous sodium sulfate and aliquots injected in a gas chromatograph equipped with an EC detector and a glass column packed with 5% OV-17 on Anakrom A.S. which undergoes initial exhaustive silanization and periodic resilanizing thereafter. As we have noted earlier (*2*), silanization is of paramount importance to achieve satisfactory chromatographic results.

While we have experienced considerable success with the isolation and chromatography of monomethyl- and dimethylarsenic present in tissue, problems were encountered in the case of inorganic arsenic, recovery levels in the neighborhood of 60% being obtained. Efforts to improve upon these early results are continuing.

In Figure 4 are shown typical chromatograms obtained from tissue samples with and without added arsenicals. The chromatogram for the tissue samples of carefully selected control animal tissue appear to be sufficiently free of chromatographic artifacts in the region of elution of the arsenicals that rather low levels of same should be cleanly measured. Some overlap is seen of the dimethylarsenic with the solvent front, but attempted further resolution is complicated by increasing the retention time and peak flattening of the co-eluted monomethylarsenic. In any event, the extent of resolution is adequate for quantitation.

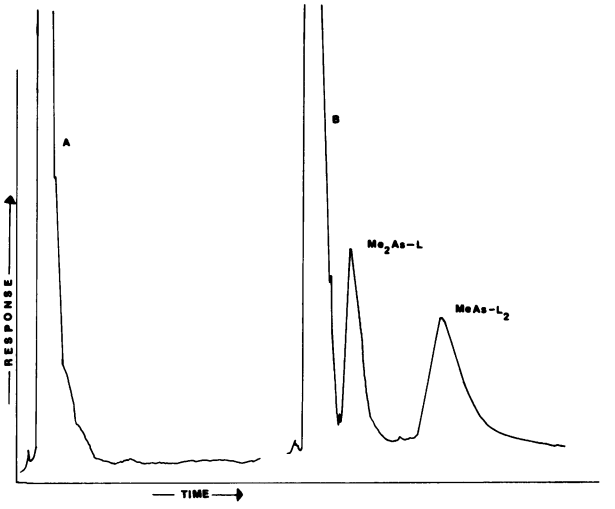

FIGURE 4. Chromatograms for methylated arsenicals in tissue by GLC: (A) liver homogenate of control animal carried through GLC procedure; (B) liver homogenate with added Me-As and Me_2-As carried through procedure. Conditions: column, 100°C; injector port, 180°C; detector (EC, ^3H foil), 190°C.

Arsenical recovery and precision (RSD) data are presented in Table 3, where it may be seen that near-quantitative recoveries with good attendant precision are readily achieved.

Planned studies involving refinement of the GLC technique as described to permit greater sensitivity include preconcentration of the benzene layers in tandem with the use of internal standards. Obvious choices as internal standards for monomethyl- and

Table 3. Recovery and precision data for tissue arsenicals by gas–liquid chromatography.

Arsenicals	Amount added, ppm	N	Recovery, % (RSD)
Mixture of:			
Methyl-As	0.9	5	98.0(±5.8)
Dimethyl-As	1.0	5	108.0(±6.3)

dimethylarsenic are the ethyl analogs.

Consideration of the two complementary but distinct approaches we are evolving for chemical speciation of arsenic in various media indicate advantages and disadvantages for one relative to the other. At this time, for example, inorganic arsenic in media by GLC poses problems to us, when such analysis is desired as part of a uniform analytical scheme. Such does not appear to be the case with FAA spectrometry, however. On the other hand, assessment of monomethyl- and dimethylarsenic is more quickly achieved via GLC, and resolution of difficulties with inorganic arsenic would then permit considering the separation scheme laid out in Figure 2 in a greatly contracted version for GLC studies, i.e., fractions A and A′ in Figure 2 are directly analyzed by GLC without further effort at generation of additional fractions but furnishing essentially the same information.

The methodologies elaborated upon in our report direct themselves to chemical speciation of arsenic in biological media, but to be perfectly rigorous it is a partial speciation approach, given limited knowledge on our part as to the possible existence of other forms of arsenic, such as the hydrides, in animal tissue.

With specific reference to the arsenic hydrides, i.e., arsine, methylarsine, dimethylarsine, and trimethylarsine, little is known concerning the generation, transport, deposition or excretion of these species in mammalian systems, so that a rational analytical approach to assess the presence of these forms in higher organisms is not immediately obvious.

Arsine and its lower alkyl organic derivatives are gaseous, labile entities, and one would have to determine the presence or absence of biochemical constraints on these forms *in vivo* before designing analytical approaches. Tertiary arsines, for example, form strong σ-bonded complexes with a number of metal ions, and the voluminous literature concerning this area has been reviewed (*5–7*). The extent to which arsines, especially tertiary arsines such as trimethylarsine, may be moved about in higher organisms as their metal complexes is essentially unknown. Tertiary arsines also show a marked propensity for quaternization to the tetraorgano ion (*8*) using an alkyl donor and, again, the extent to which this might occur *in vivo* is unknown.

Presumably one could assess the presence of free arsines in respired air of, say, experimental animals given arsenic via a respiratory chamber which has been modified to permit the assessment of the arsines by GLC, as per the technique described by Talmi (*9*). Similarly, one could assess the presence of chemically unincorporated or free arsines in tissue by closed-vessel homogenizing/microsonicating, followed by head space analysis using GLC.

The manipulation given tissue samples to apply methods such as those described by us and, in fact, most methods currently in use are done so as to liberate arsenicals from the matrices in which they occur. Consequently, no information may be gained as to the nature of the biochemical species into which arsenicals have been incorporated *in vivo*.

The nature of this incorporation has a direct bearing on the effects of arsenicals at the cellular and subcellular level and therefore the question is biochemical rather than quantitative analytical in nature. This being the case, one must adopt considerably different approaches to attacking such a problem, and these must include nondestructive techniques such as liquid or other chromatographic separations in tandem with radioisotopic tracing.

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